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(54) Title: IMPROVED PROKARYOTIC EXPRESSION OF PROTEIN

(57) Abstract

The invention herein described relates to a novel prokaryotic expression system and proteins expressed thereby. The regulated expression or alteration of the *wprA* gene from the genome of *B. subtilis* has been shown to result in enhanced secretion of selected native, heterologous or recombinant polypeptides. This alteration provides the means by which the yield of secreted polypeptide is increased compared to strains carrying a wild type copy of the *wprA* gene.

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IMPROVED PROKARYOTIC EXPRESSION OF
PROTEIN

The invention relates to a novel prokaryotic expression system and proteins expressed thereby.

5 The industrial production of proteins has, in many instances, exploited the native expression and secretory systems of micro-organisms and specifically bacteria. For example, and without limitation, the bacterium *Bacillus subtilis* is known to produce and secrete a number of proteins. One of these proteins α -amylase is of industrial importance and therefore the harvesting of this secreted
10 protein is an activity currently undertaken by industry. However, the yield of this protein is significantly reduced by protein degradation during or immediately after, or just after, passage through the cell membrane.

It therefore follows that there is a need to provide a protein expression system which enhances the production of native protein or indeed heterologous or
15 recombinant protein and more specifically enhances this production by reducing the degradation of protein.

It is also known to provide microorganisms that express and, advantageously, secrete heterologous protein i.e. proteins that are not native to that particular bacteria. This sort of system typically involves transformation of a bacterial cell
20 with heterologous DNA with a view to manufacturing or producing recombinant protein. Microorganisms such as *Escherichia coli* (bacteria) *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *Neurospora crassa* (fungi) have been used in this fashion.

The expression of heterologous protein in these primitive eukaryotes also allows some desirable eukaryotic post translational modifications to occur in these heterologous proteins leading to increases in the stability of the expressed protein and subsequent improvements in yield. More recently the use of 5 mammalian and insect cell culture systems have been developed to facilitate the expression of eukaryotic proteins that for various reasons cannot be expressed in a prokaryotic host cell.

However the cost effectiveness of producing recombinant protein still remains the major advantage offered by genetically engineered prokaryotic expression 10 systems and indeed significant advances have been made in the development of genetically engineered *E.coli* strains that increase the yield of specific recombinant proteins. The evolution of these bacterial strains has also been married with an ever increasing development of more efficient vectors adapted to optimize the expression of recombinant protein. These vectors commonly 15 contain promoter elements that can be switched on or off with ease.

However there are two major disadvantages when using *E.coli* as a means of expressing recombinant protein. Firstly the high levels of expression may lead to a precipitation of recombinant protein in the bacterial cytoplasm as "inclusion bodies". This feature was thought to be advantageous as it can provide a simple 20 means of separating the insoluble recombinant protein from the soluble endogenous *E.coli* protein. However, in reality this advantage is not a general feature of the system as in many cases the protein remains as an insoluble precipitate that can only be released into solution by using strong chaotropic agents. This presents a major problem if the protein in question is particularly 25 labile and therefore loses biochemical or biological activity upon denaturation. Secondly the expression of foreign protein in *E.coli* leads to the rapid

degradation of these proteins via an efficient proteolytic system. Therefore difficulties arise with regard to the isolation of intact recombinant protein from *E.coli* cells.

5 *E. coli* strains (TOPP series, BL21) have been engineered to allow the expression of recombinant proteins that would ordinarily be difficult to express in traditional laboratory strains of *E. coli*. However these engineered *E.coli* strains are invariably not as biologically disabled as traditional laboratory strains of *E. coli* and as a consequence require containment levels that are higher than what would normally be required.

10 The identification of alternative prokaryotic host cells and the development of means that facilitate the production of soluble, intact and biologically active protein is obviously desirable. However, notably the number of potential prokaryotic host cells is huge.

15 With a view to producing a novel protein expression system we have chosen to genetically engineer, as our example, *Bacillus subtilis* in order to provide an expression system that overcomes the problems of yield associated with prior art systems. We have focused our attention on providing a bacterial expression system that produces and ideally secretes proteins (native and/or heterologous and/or recombinant) into the culture medium because this system enables 20 purification of the manufactured protein due to the absence of contaminating endogenous bacterial proteins and other macromolecules.

A number of *B. subtilis* genes encoding secreted proteases have been identified. For example, without limitation, *aprE*, *nprE*, *bpf*, *mpr*, *epr*, *nprB* and *vpr* genes of *B. subtilis* encode extracellular proteases. These proteases are secreted into

the culture medium and deletion of them from the *B. subtilis* genome reduces extracellular protease activity to less than 1% of wild type strains. Despite this fact experimental evidence suggests that *B. subtilis* strains deficient for extracellular proteases still show significant loss of secretory protein production

5 through proteolytic degradation.

The identification of a cell wall-associated protease has lead us to investigate whether it has a role in expression and secretion of native and/or heterologous and/or recombinant protein in *B. subtilis* and whether or not the protease plays a major role in determining the levels of secretory protein production.

10 The wall protease A (*wprA*) gene encodes a 96 kDa polypeptide containing a signal peptide, propeptide and a protease which upon synthesis and export gives rise to two cell wall-bound proteins, CWBP23 and CWBP52 (Figure 1). The CWBP52 polypeptide has protease activity that is inhibited with PMSF, an inhibitor of serine proteases. The deletion of *wprA* does not result in any

15 noticeable phenotype in terms of growth rate, cell morphology, sporulation or motility.

The *wprA* is characterised by the following characteristics; the polypeptide is cell-wall associated and is expressed during both exponential and stationary growth phases.

20 We decided to investigate the phenotype of a *wprA* deletion strain with particular reference to the secretion of heterologous protein.

Using homologous recombination, we have generated a strain in which the *wprA* gene from the *B. subtilis* genome has been placed under the control of an inducible promoter. As mentioned above, this *wprA* controllable strain has no

apparent phenotype, even in the absence of an inducer. Surprisingly, however, when the yield of a native bacillary model protein, the α -amylase from *Bacillus licheniformis* (AmyL) was compared in wild-type and *wprA* null strains there was an approximate 25% increase in the amount of α -amylase detected at the 5 end of exponential growth, see figure 2A. The yield increased further to 41% after prolonged incubation (Figure 3). Knocking out the *wprA* gene also resulted in an increase in the yield of an engineered α -amylase, AmyLQS50.5, when compared to the wild-type *wprA* strain (figure 2B). This suggests that switching off or deleting the *wprA* gene significantly enhances production of 10 secreted homologous and heterologous proteins in *B. subtilis*.

The importance of the *wprA* gene with respect to the secretion of native, heterologous or recombinant protein is also shown by experiments that attempted to identify additional genes involved in the secretory pathway. We have used a *B. subtilis* strain, CJ278, transformed with a vector expressing a 15 chimeric α amylase gene, please see materials and methods for details of the chimeric α amylase gene and *B. subtilis* strain construction. This strain showed decreased chimeric α -amylase secretion in comparison to the level of secretion by a wild type strain expressing wild type α amylase. We have used transposon mutagenesis using the mini-Tn 10 delivery vector pIC333. The integration of 20 this transposon into the *B. subtilis* genome is random. The screen involved the identification of integration mutants that show elevated secretion of chimeric α -amylase. The mutant strains thus identified were analysed further by rescuing the plasmid DNA from the mutant strain and sequencing the flanking regions around the transposon integration site to determine the region of the *B. subtilis* 25 genome in to which the transposon had integrated. The sequence of the plasmid rescued from the transposon mutant TK108 shows that the transposon had

inserted into the *wprA* gene at position 2059.

It is therefore apparent that the absence of the WprA facilitates the secretion of native, heterologous or recombinant protein from *B.subtilis*.

It is therefore an object of this invention to develop a means of expressing 5 recombinant protein in a prokaryotic expression system that allows the production of polypeptides in a biologically active form and at a high concentration.

It is a further object of the invention to develop a prokaryotic expression system 10 that enables the secretion of recombinant protein into the culture medium to facilitate the purification of intact recombinant protein that retains biological activity.

According to a first aspect of the invention there is provided a bacterial strain 15 wherein the *wprA* gene or its corresponding promoter has been altered by deletion and/or insertion and/or mutation and/or substitution so that either production of the said gene product is prevented or the gene product is non-functional to the extent that the use of the strain to produce native, heterologous or recombinant protein is facilitated.

In a preferred embodiment of the invention said bacterial strain, prior to said alteration, is wild-type for said *wprA* gene.

20 In yet a further preferred embodiment said bacterial strain is a Gram-positive bacterial strain.

In yet a still further preferred embodiment said bacterial strain is of the genus

Bacillus.

Reference hereto the term bacterial strain includes reference to any bacterial strain but ideally a Gram-positive bacterial strain and, more ideally, but not obligatory, a bacterial strain of the genus *Bacillus*.

- 5 It will be apparent to those skilled in the art that where heterologous protein is to be produced the said bacterial strain will be transformed so as to include DNA encoding at least one selected native and/or heterologous and/or recombinant protein.

In a preferred embodiment of the invention the said strain is manipulated so that

- 10 at least a part of the *wprA* gene is deleted. Ideally a significant amount of the gene is deleted, but in certain aspects of the invention the pre-sequence, or a part thereof, or alternatively, the pro-sequence, or a part thereof, or alternatively the serine protease sequence, or a part thereof is deleted. Alternatively, a selected alternative part of the gene may be deleted or a combination of selected
- 15 parts may be deleted.

In an alternative embodiment of the invention genetic material may be inserted into the *wprA* gene at at least one selected location with a view to preventing expression of the gene or synthesis of at least a part of the functional protein product.

- 20 Alternatively, at least one selected point mutation may be provided in the said gene with a view to either preventing synthesis of the protein or preventing expression of the gene. For example, and without limitation, the reading frame of the gene may be altered so as to encode a stop codon thus preventing synthesis of a functional protein.

In a yet further preferred embodiment of the invention said *wprA* gene is altered by way of modification of an expression control sequence, ideally a promoter such that the promoter is made responsive to a specific signal, for example, the *wrpA* gene may be placed under the control of an inducible promoter such that 5 expression of the *wrpA* gene product may be selectively controlled.

In a preferred embodiment of the invention the bacterial strain is of the genus *Bacillus* and is ideally the species *Bacillus subtilis* or its close relatives such as *B. amyloliquefaciens*, *B. licheniformis* and *B. stearothermophilus*.

It is noted that the *wprA* gene encodes a protein with specific domains attributed 10 to its derived amino acid sequence. The polypeptide can be divided into a signal peptide, propeptide and protease polypeptide that have been accurately defined in Margot and Karamata (Microbiology 1996 142 3437-3444). The signal peptide is involved in targeting the *wprA* gene product to the secretory apparatus necessary for translocation across the cell membrane. The propeptide 15 is likely to be a chaperone-type molecule involved in folding and maturation of the CWBP52 protein into a biologically active form. The propeptide is stable and may perform other important functions. The protease polypeptide will require the presence of both the pre and pro-sequences to be effectively targeted and to function efficiently.

20 According to a second aspect of the invention there is provided a bacterial strain, preferably of the genus *Bacillus*, and ideally of the species *B. subtilis* having a deletion in at least part of the sequence represented in Figure 1 from nucleic acid base pair +154 to +247 inclusive, or a corresponding part of a homologous gene.

In a preferred embodiment there is provided a *B. subtilis* strain deleted for a part of the *wprA* gene encoding the signal peptide of the coding sequence of the WprA precursor protein.

According to a third aspect of the invention there is provided a bacterial strain 5 preferably of the genus *Bacillus* and ideally *B. subtilis*, having a deletion of, at least a part of, the sequence represented in Figure 1 from nucleic acid base pair +154 to +1392 inclusive, or a corresponding part of a homologous gene.

In a preferred embodiment the said bacterial strain may be modified, 10 additionally or alternatively, by deletion of, at least part of, the DNA sequence presented in Figure 1 from +247 to +1392, or a corresponding part of a homologous gene.

In yet a further preferred embodiment said *B. subtilis* strain is deleted for that part of the *wprA* gene encoding, at least a part of, the polypeptide CWBP23.

According to a fourth aspect of the invention there is provided a bacterial strain 15 preferably of the genus *Bacillus* and ideally the species *B. subtilis* having a deletion of, at least a part of, the sequence represented in figure 1 from nucleic acid base pair + 1392 to +2835 inclusive, or a corresponding part of a homologous gene.

In a preferred embodiment there is provided a *B. subtilis* strain deleted for that 20 part for the *wprA* gene encoding, at least a part of, the CWBP52 polypeptide.

In yet a further preferred embodiment of the invention said bacterial strain is deleted for at least part of the sequence represented in Figure 1 from nucleic

acid base +247 to + 2835.

In yet a further preferred embodiment of the invention there is provided a *B.subtilis* strain deleted for part of the *wprA* gene encoding either or both the proposed propeptide (CWBP23) or the serine protease (CWBP52).

- 5 In yet a further preferred embodiment the said strain includes a deletion in that part of the gene encoding a polypeptide that is a serine protease.

In a further aspect of the invention there is provided a method for producing a desired polypeptide, wherein a microbial strain as indicated above is used for the production of said polypeptide by growing said strain under conditions
10 conducive to the production of said polypeptide of interest, and recovering said polypeptide of interest.

It is envisaged that the polypeptide of interest may be endogenous or heterologous to the strain in question.

According to an embodiment of this aspect said microbial strain is used as a
15 host into which a polynucleotide construct encoding said polypeptide of interest is brought in a functional manner by which said strain is capable of expressing said polypeptide.

The polynucleotide construct may be transferred into the strain by any method known in the art, such as transformation, conjugation, or protoplast
20 transformation. The construct may be a plasmid or any other vector suitable for the specific method used for introducing said polynucleotide construct into the microbial cell.

In the cell said construct may be present on a plasmid or integrated into the chromosome of said strain. Furthermore, it may be present as a single copy or in multiple copies provided by either amplification or by multiple integrations.

5 The polypeptide may be a peptide or protein of any type, especially an industrial enzyme. Said enzyme may be any enzyme that can be produced in a strain according to the invention, such as a carbonyl hydrolase, carbohydrase, protease, lipase, amylase, cellulase, oxido reductase, glucoamylase, or esterase.

In essence the invention provides a bacterial strain, ideally a *B. subtilis* strain, altered by mutation, substitution, insertion or deletion either entirely or in part, 10 for the *wprA* gene, or a homologue thereof, which gene encodes a cell wall-associated serine protease. It is surprising that given the presence of additional extracellular protease genes in *B. subtilis* that deletion of the single copy *wprA* gene should result in a significant effect on the production of both endogenous and heterologous recombinant protein.

15 An embodiment of the invention will now be described by way of example only with reference to the following figures wherein:

Figure 1 shows the nucleotide sequence of the region of the *B. subtilis* genome containing the *wprA* gene and amino acid sequence of its product WprA;

Figure 2A represents yields of α -amylase released into culture medium. Closed 20 symbols represent growth and open symbols α -amylase activity. *B. subtilis* strains KS408 (■), KS408 *wprA* :: pMutin2 with (◆) or without (●) IPTG (10 mM);

12A

.....shows precursor (p) and mature(m) immunoprecipitated from whole culture samples and the bottom panel mature AmyL (m) released into culture medium. Quantification by phospho-imaging of the different forms of AmyL at time intervals following the chase; AmyL precursor () and mature AmyL in whole cultures samples (♦), and mature AmyL released into growth medium (•). The amount of each form of AmyL is expressed as a percentage of the total AmyL (precursor + mature) synthesized during the pulse;....

Figure 2B is similar to the experiment described in Figure 2A but the *B. subtilis* strains are expressing a recombinantly manufactured chimeric α -amylase (AmyLQS50.5). Experimental details relating to induction of the *wprA* gene product are as in Figure 2A and are described in detail in the materials and 5 methods;

Figure 2C is a diagrammatic representation of the construction of a *B. subtilis* strain encoding an inducible *wprA* gene. Closed flags represent the native *wprA* promoter (*PwprA*) and open flags the IPTG- inducible promoter (*Pspac*).
10 Ori Ec; *E. coli* origin of replication; A represents the sub-cloning of a *wprA* PCR 5' fragment into the BamH1 site in pMutin2; B represents a single cross-over event between pM2*wprA*FP and the *B. subtilis* *wprA* gene; C represents the integration of pM2*wprA*FP into the *B. subtilis* chromosome by homologous recombination; and D represents the structure of the *B. subtilis* chromosome after the integration event.

15 Figure 3 represents a comparison of AmyL production in a wild type *B. subtilis* strain and a strain with a *wprA* gene product under the control of an IPTG inducible promoter in the absence or presence of IPTG. Cultures of *B. subtilis* were grown to stationary phase and AmyL activity was compared during exponential growth phase and after approximately 30 hours in stationary phase;
20 Figure 4 represents the secretion kinetics of AmyL from exponentially growing *B. subtilis* in the presence and absence of the *wprA* gene products. Representative data from pulse-chase experiments carried out on strains KS408 and KS408*wprA* ::pMutin2+/- 10mM IPTG. (A) Autoradiographs of pulse-chased AmyL following immunoprecipitation and SDS-PAGE. The top panel

Figure 5 represents cell-associated degradation of AmyL as determined by subtracting the data for the released mature AmyL from that obtained in the whole culture samples. The amount of AmyL at each interval is expressed as a percentage of the maximum amount of AmyL (precursor + mature) synthesized 5 during the pulse;

Figure 6 represents the stability of AmyL in spent culture medium at 4°C. (A) α -Amylase activity at time intervals in the absence (■) or presence (◆) of 10mM EDTA. (B) western blots of AmyL in spent culture medium at time intervals in the absence and presence of 10mM EDTA; and

10 Figure 7 represents the transcriptional activity of the *wprA* gene using the *wprAΔ-lacZ* transcriptional fusion. Growth (closed symbols) and β -galactosidase activity(open symbols) were measured in cultures of *B.subtilis* KS408 (■) and KS408*wprA* ::pMutin2 with (◆) or without (●) IPTG (10mM)

Table 2 shows the production of the AmyL α -amylase by *B. subtilis* in the 15 absence or presence of the *wprA* gene product in a very nutritious, industrial type medium in an extended batch fermentation. Each strain was grown for approximately 7 days at 37°C and α amylase activity was measured in the supernatant at the end of this period. Experimental details are given in the materials and methods.

20 MATERIALS AND METHODS

The initial analysis of the involvement of the *wprA* gene product in secretion of endogenous and heterologous recombinant protein dealt with the construction of a *B. subtilis* strain in which the single copy *wprA* gene promoter was

substituted by an IPTG inducible promoter. In the absence of IPTG the expression of the *wprA* gene is repressed. Upon addition of IPTG the *wprA* gene is induced.

Alternatively the *wprA* gene can be entirely or partially deleted from the 5 *B.subtilis* genome as detailed in preceding description and the following methods.

Bacterial strains

The bacterial strains used are shown in table 1.

Table 1. Bacterial strains

Strain	Comments
<i>E. coli</i> XL1-Blue	-
<i>B.subtilis</i> DN1885 xylR::pKS405B	Encodes chimeric α -amylase AmyLQS50.5
<i>B. subtilis</i> DN1885 xylR::pKS408	Encodes wild type α -amylase, AmyL
<i>B. subtilis</i> DN1885 xylR::pKS405B, wprA:: pM2 wprAFP.	<i>B. subtilis</i> DN1885 xylR:: pKS405B with IPTG-inducible wprA
<i>B. subtilis</i> DN1885 xylR:: pKS408	<i>B. subtilis</i> DN1885 xylR::pKS408

wprA::pM2 wprAFFP	with IPTG-inducible wprA
	α -amylase (<i>amyE</i>) negative
<i>B.subtilis</i> DN1885 Novo Nordisk	derivative of <i>B.subtilis</i> RUB200
J.of Bacteriology 172:4315-4321,	
1991	

Growth media

B.subtilis and *E.coli* were maintained on antibiotic medium number 3 (Difco) solidified with 1.5% w/v agar and containing 1% w/v soluble starch. Batch cultures were grown in 2xYT broth which contained; tryptone (1.6%w/v), yeast extract (1.0%w/v) and NaCl (0.5%w/v). Where required antibiotics were included in the growth media at the following final concentrations: 5 chloramphenicol g/ml, ampicillin g/ml and erythromycin g/ml. Xylb^{gal} (0.2%w/v) was added to induce the synthesis of α -amylase from a xylose-inducible promoter. The comparison of α -amylase production in *B.subtilis* wild type and 10 a strain deleted for the *wprA* gene product was also done in an industrial type medium containing potato starch (100g/l), barley flour (50g/l), BAN 5000 SKB (0.1g/l), sodium caseinate (10g/l), soy bean meal (20g/l), Na₂HPO₄.12 H²O (9g/l) and pluronic (0.1g/l). For the wild-type strain the medium was supplemented with 6 g/ml chloramphenicol and 0.2% xylose. For the *wprA*

deletion strain medium was supplemented with 6 g/ml chloramphenicol 5 g/ml erythromycin and 0.2% xylose.

DNA manipulations and bacterial transformation

Restriction digestion, DNA fragment purification, ligation and transformation of *E.coli* were carried out as described previously (Sambrook et al., 1989). Chromosomal DNA was isolated from *B.subtilis* using the IGI Genomic extraction kit (Immunogen International). PCR was carried out with Taq DNA polymerase (Appligene) using *B.subtilis* DN1885 chromosomal DNA as the template. Plasmid DNA was purified from *E.coli* and *B.subtilis* with the Tip-100 plasmid extraction kit (Qiagen). Oligonucleotide primers for PCR were synthesized using a Beckman Oligo 1000. *B.subtilis* was grown to competence and transformed with integrative plasmids.

α -Amylase assay

The quantity of secreted α -amylase was quantified using the Phadebas α -amylase assay kit (Kabi Pharmacia). The cells from culture samples were pelleted by microcentrifugation and the α -amylase activity in the supernatant determined as described by the manufacturer.

Construction of a Strain Encoding An Inducible *wprA*

To determine whether the products of the *wprA* gene are involved in the co-or post-translocational degradation of AmyL, we constructed a strain of *B.subtilis* in which an intact copy of the gene is under the control of the isopropylthio- β -D- galactoside (IPTG) -inducible Pspac promoter. The constructs were made using the pMutin2 integration vector. A 357-base pair DNA fragment

corresponding to the 5' end of the *wprA* gene was amplified by PCR from *B.subtilis* KS 408 chromosomal DNA using oligonucleotide primers WPR-F (5' GCGCGCGCGGATCCGGATAACATGAAACGC 3') and WPR-R (5' GCGCGCGCGGATCCCCATCCTCCGCTGTG 3'). This fragment was 5 cloned into the unique BamH1 restriction site of pMutin2 using *E.coli* XL1-Blue as the host.

The resultant plasmid, pM2wprA^{FP}, was used to transform *B.subtilis* KS408 to produce strain KS408 *wprA* ::pMutin2. Since the *wprA* gene of KS408 *wprA* ::pMutin2 is under the control of the Pspac promoter, its expression can be 10 controlled by the presence or absence of IPTG. Additionally, a transcriptional fusion (*wprA* Δ-lacZ) was created between the native *wprA* promoter and lacZ to allow the expression of *wprA* to be monitored via β-galactosidase activity, Figure 7.

Construction of a WprA Negative Strain By Deletion in the *B.subtilis* DN1885
15 *wprA* Gene

Plasmid pCJ791, encoding a N-terminal fragment (bp 133 to bp 615) and a C-terminal fragment (bp 2364 to bp 2781) of the *wprA* gene from *B.subtilis* DN1885, was constructed in four steps:

i) a 382 bp N-terminal fragment of the *wprA* gene was amplified by PCR
20 from *B.subtilis* DN1885 using oligonucleotide primers
CLJe7
(5'-GGAATTCCAAAGCTGCAGCGGCCGGCGCG-3'),
and CLJe8
(5'-GAAGATCTCGTATACTTGGCTTCTGCAGCT-3').

This fragment had a EcoRI restriction site at the 5'-end and a BglII restriction site at the 3'-end. Simultaneously, a 419 bp C-terminal fragment of the *wprA* gene was amplified by PCR from *B. subtilis* DN1885 using oligonucleotide primers

5 CLJe9

(5'-AGATCTGGTCAACAAGCTGGAAAGCACTC-3')

and CLJe10

(5'-CCCAAGCTTCGTGACGTACAGCACCGTTCCGGC-3').

This C-terminal fragment had a BglII restriction site at the 5'-end and a
10 HindIII restriction site at the 3'-end.

ii) The two DNA fragments, encoding the N- and C-terminal sequences of the *wprA* gene, were digested with BglII restriction enzyme and ligated to form a fragment of 801 bp. Using oligonucleotide primers CLJe7 and CLJe10, the 801 bp fragment was amplified by PCR from the ligation mixture. The amplified 801 bp fragment was digested with EcoRI and HindIII restriction enzymes.

15

iii) A 4.4 kbp EcoRI to HindIII fragment from plasmid pSJ2739 (described in patent application WO 96/23073, figure 6) was purified and used as vector for the 801 bp fragment. This plasmid is based on the pE194 origin of replication which means that the replication of the plasmid is
20 temperature sensitive.

iv) The two EcoRI to HindIII fragments (801 bp and 4.4 kbp) were ligated and plasmid pCJ791 was obtained by selecting for resistance to erythromycin at 28°C using *B. subtilis* DN1885 as host strain.

Plasmid pCJ791 was integrated into the chromosome of *B.subtilis* DN1885 by selecting for resistance to erythromycin at 37°C. Since pCJ791 is based on the pE194 origin of replication, transformants were selected in which the plasmid had integrated into the chromosome by homologous single crossover recombination between one of the plasmid *wprA* sequence and the corresponding chromosomal *wprA* sequence. Two types of integrant strains could be the result of the integration event, i) the integrated plasmid followed by the wild-type *wprA* gene or ii) the wild-type *wprA* gene followed by the integrated plasmid. For the construction of a clean *wprA* deletion strain, both types of integrant strain could be used. Hence, the integration event was not investigated further.

A clean *wprA* deletion strain was then constructed by homologous single crossover resulting in release of the integrated plasmid. There were two ways in which the plasmid could be released from the chromosome i) by the same recombination as the plasmid was integrated or ii) by recombination between the sequence that was not involved in the integration event. For the first case, the resulting strain would have a wild-type *wprA* gene on the chromosome. If the second case occurs, the resulting strain would have a deleted *wprA* gene on the chromosome and, by that, the wanted event. In order to release the integrated plasmid, twelve transformants were inoculated in TY-medium without selection and cultivated at 28°C over night. The cultures were once again inoculated in fresh TY-medium and cultivated at 28°C over night. After three rounds of inoculation, the cultures were spread on LB-plates without selection and, subsequently, the obtained colonies were screened for sensitivity to erythromycin. Twenty-four colonies sensitive to erythromycin (Erm^R) were checked for the presence of a deleted *wprA* gene by PCR directly on colony

using oligonucleotide primers CLJe7 and CLJe10. Four of these *Erm*^s colonies had a deleted *wprA* gene on the chromosome. The authenticity of the *B. subtilis* DN1885Δ*wprA* strain was confirmed by Southern Blot Hybridisation.

Isolation of a *wprA* mutant with increased level of secreted chimeric α -amylase

5 In a previous study where the aim was to investigate how the net charge of proteins affected their passage through the negatively charged cell wall in *Bacillus subtilis* it was observed that both wild type AmyL (α -amylase from *Bacillus licheniformis*) and chimeric variants are subject to co-and/or post-translocational degradation. Protease(s) responsible for this degradation are
10 likely to be associated with the cytoplasmic membrane or cell wall, since the proteolytic degradation occurs on the outer surface of the cytoplasmic membrane ("Construction and use of chimeric α -amylase to study protein secretion in *B. subtilis*" PhD thesis by Keith Stephenson, University of Newcastle Upon Tyne, 1996; "Secretion of chimeric α -amylase from *Bacillus subtilis*" PhD thesis by Christina Lund Jensen, Technical University of Denmark, 1997). In a search of factors involved in this degradation, a screening system based on the *B. subtilis* strain CJ278, expressing the chimeric α -amylase (AmyLQS55-6) was set up. A mutant library was prepared by transposon mutagenesis, and subsequently screened for mutants with increased halo
15 formation on amylase screening plates.

Construction of a chimeric α -amylase, AmyLQS55-6:
In the following an overview of the steps involved in the construction of the chimeric α -amylase AmyLQS55-6 is given. A detailed description is given in: "Secretion of chimeric α -amylases from *Bacillus subtilis*." Ph.D thesis by

Christina Lund Jensen, Technical University of Denmark, 1997.

The chimeric α -amylase, AmyLQS55-6 was constructed by swapping specific blocks of the mature portion of the α -amylase from *B. licheniformis* (AmyL) with the corresponding blocks from the α -amylase from *B. amyloliquefaciens* 5 (AmyQ) or from *B. stearothermophilus* (AmyS). The individual DNA blocks were constructed by using a PCR-based in vitro gene splicing method, the SOE method (splicing by overlap extension, Horton et al. Gene 77, 61-68, 1989). The amyL gene has a unique PstI site located within the signal sequence and a unique HindIII site 3' to the transcription terminator. The amyLQS55-6 gene 10 was therefore designed as in-frame PstI to HindIII DNA fragments encoding the mature part of the α -amylase. The amyLQS55-6 gene was divided into 3 separate DNA blocks, with block 1 covering a PstI-BamHI fragment, block 3 a KpnI-SalI fragment and block 4 a SalI-HindIII.

15 The properties of each block is given below, with the base numbers calculated in relation to the start codon in each gene.

Block 1: bp 79-132 amyL, bp 151-174 amyS, bp 157-198 amyQ, bp 199-213 amyL,

Block 3: bp 562-993 amyL, bp 1018-1095 amyS, bp 1072-1095 amyL

Block 4: bp 1096-1221 amyL, bp 1237-1419 amyS, bp 1411-1542 amyQ, bp 20 1537-1798 amyL

Between block 1 and 3 is a wild type amyL block covering bp 214-561.

The individual blocks were cloned into pUC19 in the correct order to produce the PstI to HindIII gene fragment encoding the mature AmyLQS55-6 protein.

For assembly of the individual blocks advantage was taken of the unique restrictions sites generated at their ends (created by the SOE method).

Expression of the chimeric α -amylase, amyLQS55-6

The amyLQS55-6 gene was integrated into the *B.subtilis* chromosome by

5 homologous recombination between a plasmid encoded and a chromosomal encoded copy of the *xylR*-gene. The assembled amyLQS55-6 gene was cloned into plasmid pCJ92. Plasmid pCJ92 is derived from pSX63 which encode for a xylose-inducible promoter system (for detailed information about the construction of plasmid pCJ92; Secretion of chimeric α -amylases from *Bacillus subtilis*. Ph.D thesis by Christina Lund Jensen, Technical University of Denmark, 1997). The EcoRI to BglII fragment of the amyLQS55-6 gene encoded by pCJ92 was cloned into pUC19 EcoRI and BamH1 restriction sites in *E.coli* SJ2, resulting in plasmid pCJ272. Plasmid pCJ272 does not contain an origin of replication that is functional in *B.subtilis*. The integration plasmid, 10 pCJ272, was introduced into the *B.subtilis* strain DN1885 and transformants were obtained by selection for chloramphenicol resistant colonies. The plasmid was integrated into the chromosome of DN1885 by a single, homologous recombination between the plasmid encoded and chromosomally encoded 15 copies of the *xylR*-gene.

20 The integration of the α -amylase expression cassette into the chromosome of DN1885 resulted in a stable system which allowed the production of α -amylase to be induced in the presence of xylose.

Screening system:

The screening system for identification of mutants with an improved secretion

of α -amylase is based on the *B. subtilis* strain CJ278 (DN1885 xylR::pCJ272) harbouring the gene encoding the chimeric α -amylase AmyLQS55-6. In comparison to the wild type AmyL, the level of α -amylase secretion from strain CJ278 is about 1%, meaning that CJ278 gives rise to colonies with a small and 5 well-defined halo of starch degradation on plates. Therefore, it was considered an ideal candidate for screening of yield mutants.

Mutagenesis protocol:

For transposon mutagenesis of strain CJ278, the mini-Tn10 delivery vector pIC333 was used (Steinmetz, M. and Richter, R. 1994. J. Bacteriol. 172:5019). 10 Outside the transposon, this plasmid carries a modified transposase gene conferring relaxed target specificity, a thermostensitive origin of replication and an erythromycin resistance gene for selection at permissive temperatures. The 2.2 kbp transposon encodes the spectinomycin resistance gene and the pUC8 origin of replication, allowing replication in *E. coli*. Plasmid pIC333 was 15 transformed into strain CJ278 and erythromycin resistant transformants were inoculated to TY-medium supplemented with 0.4% glucose and spectinomycin (120 μ g/ml) and grown over night at 28°C. The over night culture was diluted 1/100 in TY-medium supplemented with 0.4% glucose and spectinomycin (120 μ g/ml). After 3 hours of cultivation the temperature was shifted to 37°C (which 20 is the restrictive temperature) and the culture was cultivated for an additional 4 hours. Aliquots of the culture were plated on LB-amylopectin (coupled to Ciba-crone red) plates supplemented with 0.4% glucose, 0.01 M phosphate pH 7, 0.2% xylose and 120 μ g/ml spectinomycin and incubated over night at 37°C. Colonies with a distinctly larger halo, indicating a higher amount of secreted α - 25 amylase, appeared with a frequency of 1/150. One such transposon mutant,

forming a larger halo of starch degradation than the parent strain, was strain TK108.

The mini-Tn10 transposon and its flanking regions from strain TK108 were rescued, taking advantage of the pUC origin of replication present in the 5 transposon. The TK108 chromosome was totally digested with EcoRI, and religated with T4 DNA ligase. The ligation mixture was transformed into *E.coli* SJ2 (Diderichsen et al, 1990. J. Bacteriology 172, 4315-4321), selecting for spectinomycin resistance. Plasmid DNA from spectinomycin-resistant transformants was used for DNA sequencing. The DNA sequences was 10 determined by the dideoxy chain termination method (Sanger et al 1997) and by using mini-Tn10 specific primers: 5'- CCA ATA CGC AAA CGC CCT CTC- 3' and 5'- TAG TGA CAT TTG CAT GCT TC- 3', which correspond to position 137-117 and 2181-2200, respectively, on the mini-Tn10 transposon sequence.

15 The sequence of the plasmid rescued from transposon mutant TK108 shows that the transposon had inserted into the *wprA* gene, at position 2059.

Results and Discussion

The development of efficient alternative methods of native, heterologous or recombinant protein manufacture is obviously desirable. It is apparent that not 20 all heterologous protein can be produced in a soluble, biologically active form therefore methods that facilitate the production of such proteins are continuously being designed.

We have taken the approach to develop the genus *Bacillus* and its close relatives as an alternative host cell for the production of the both native and heterologous

and recombinant protein. These bacteria have considerable advantages over other species due to their ease of growth in batch cultures and their rapid rate of cell division and furthermore their ability to secrete proteins into the culture medium at high concentrations. Furthermore, we have decided to focus on the 5 development of an expression system that produces secreted soluble protein into the culture medium to facilitate the purification of such proteins from contaminating endogenous bacterial proteins and other macromolecules. A major problem with this methodology is that many bacterial systems actively secrete proteases into the culture medium that degrade proteins in the immediate 10 environment of the bacterial cells. Some *B.subtilis* strains have been engineered to delete these genes from the bacterial genome to reduce the loss of protein through proteolytic activity. However, this can still lead to reduced yields of intact protein due to, amongst other things, the release of intracellular proteases into the growth media as strains that are multiply deficient in extracellular 15 proteases become prone to lysis thereby releasing cellular contents into the surrounding growth media.

Additionally, we have undertaken pulse-chase labelling experiments to identify the sites at which proteolysis of secreted proteins occurs. The amount of mature Amy L (a model secreted protein) released into the culture medium can be seen 20 to increase with time until it reaches a constant level. This level represents only approximately 25% of the total Amy L synthesized, an amount consistent with the AmyL remaining in whole culture samples. This means that 75% of the initially synthesized AmyL is degraded. By determining the proportion of AmyL that remains cell-associated at each time point after the addition of the 25 chase solution, by subtracting the amount of AmyL released from that observed in whole culture samples, it was possible to determine that AmyL degradation

occurred in a cell-associated location and within 7 minutes of the addition of chase, Figure 6. This data suggests that the observed degradation of AmyL occurs during or shortly after translocation across the membrane and in a cell-associated location.

5 The *wprA* gene of *B. subtilis* encodes a cell wall-associated serine protease. The *wprA* gene product is composed of a presequence (signal peptide) to assist in targeting the protease to the secretory apparatus, a prosequence which produces a stable 23kDa protein product most likely with chaperone type activity and a 52kDa serine protease. We have engineered the *wprA* gene by placing it under
10 the control of an IPTG inducible promoter element. This allows the expression of *wprA* to be strictly regulated, simply by the presence or absence of IPTG in the growth medium. When a *B. subtilis* strain which showed decreased α -amylase production is randomly mutagenised with the mini Tn 10 transposon, integrant mutants with enhanced α -amylase production were identified.
15 Sequence analysis of rescued Tn 10 DNA revealed the integration site to be the *wprA* gene. We have also created a *B. subtilis* strain that has been genetically engineered to completely delete the *wprA* gene from the genome.

We have taken wild type *B. subtilis* strain DN1885 *xylR::pKS405B* transformed with a chimeric α -amylase gene and strain DN1885 *xylR::pKS408* which is
20 transformed with wild-type α -amylase and replaced the *wprA* promoter sequence with an IPTG inducible promoter contained in the plasmid pM2*wprA*FP (see materials and methods).

The activity of secreted wild-type α -amylase was assessed in *B. subtilis* cultures in the presence or absence of 10mM IPTG. A culture in which *wprA* was
25 expressed from its native promoter was used as a controlled culture. Figure 2A

indicates that the growth rate and kinetics of a *B. subtilis* strain is not significantly affected by the absence of WprA protein (no IPTG added), as measured by culture optical density. However, in the absence of 10mM IPTG there is approximately a 25% increase in α -amylase activity in the culture 5 medium compared to the wild-type strain in which the *wprA* gene is expressed from its native promoter (Figure 2A). Figure 2B indicates a comparable effect on the production of a chimeric α -amylase.

The yield of native α -amylase in the culture medium was also assessed in stationary phase cultures of *B. subtilis* wild-type or IPTG-inducible *wprA* genes, 10 (figure 3). The strains were grown for approximately 39 hours at which time cultures had been in stationary phase for approximately 30 hours. In the absence of IPTG the yield of α -amylase in the culture medium had increased by approximately 40% when compared to a strain expressing *wprA* from its native promoter. In contrast, the yield of α -amylase from KS408 *wprA* ::pMutin2 in 15 the presence of IPTG (*wprA* on) was lower and on transition to stationary phase the yield of α -amylase was 95% that of KS408.

Additionally, when strains are grown in a rich industrial type medium in an extended batch fermentation culture, there is approximately a 78% increase in α -amylase activity in the absence of the WprA protein.

20 These data demonstrate that expression of *wprA* markedly influences the yield of released α -amylase.

We have additionally used coupled pulse-chase and immunoprecipitation techniques to investigate the secretion kinetics of AmyL in KS408 and KS408 *wprA*::pMutin2. Cultures were grown to exponential phase (OD₆₀₀ ~ 0.6) and

pulse-chased with L-(³⁵S) methionine. Following immunoprecipitation and subsequent SDS-PAGE, both precursor and mature forms of AmyL were visualised by autoradiography, Figure 4. In the case of KS408, the processing of the AmyL precursor to the mature form was rapid; in samples taken 5 immediately following the chase (0 min) only 27% of the total AmyL (precursor + mature) synthesized during the pulse was in precursor form, Figure 4. Processing was complete by 5 min post-chase when all the α -amylase was in mature form. The amount of mature AmyL in the whole culture sample (cells + growth medium peaked at 1 minute, after which time it declined until it 10 reached a constant level of approximately 25% of the maximum detected, representing a significant loss of newly synthesized α -amylase during or shortly after translocation across the cytoplasmic membrane.

The involvement of the WprA protein in the secretion of protein from *B. subtilis* is also confirmed by the mutant screen undertaken to identify mutated strains 15 that show enhanced secretion of a chimeric α -amylase. The mini-Tn 10 transposon randomly integrates into bacterial genomic DNA thereby creating insertional mutations if the transposon does not integrate in an essential gene. The mutant strain TK108 shows increased secretion of chimeric α -amylase when compared to a wild type control strain. The mini-Tn 10 delivery vector 20 was recovered from TK108 genomic DNA and the flanking regions surrounding the vector was sequenced to determine the site of integration. The transposon had integrated at position 2059 of the *wprA* gene. The disrupted *B. subtilis* strain TK108, showed elevated α amylase secretion as monitored by the size of the halo produced around TK108 when compared to CJ278(wild type) on starch 25 agar plates.

In conclusion we have shown that switching off or disabling or deleting the

single copy *wprA* gene significantly increases the yield in the culture medium of native, heterologous or recombinant proteins from *B. subtilis*. Importantly *B. subtilis* extracellular proteases are still actively secreted into the growth medium indicating that a major contributing factor in the production of 5 increased yields of secreted α -amylase is removal of the cell wall associated protease encoded by the *wprA* gene.

Table 2

Relative yields of α -amylase in wild-type and *wprA* IPTG-inducible *B. subtilis* strains.

<i>B. subtilis</i> DN1885 xylR::pKS408	<i>B. subtilis</i> DN1885 xylR::pKS408 <i>wprA</i> ::pM2 wprAFP
100	178

CLAIMS

1. A method for producing a native and/or heterologous and/or recombinant polypeptide from a microbial strain having a deletion and/or insertion and/or mutation and/or substitution in a *wprA* gene or its corresponding promoter such that the production and/or function of the gene product encoded by the *wprA* gene is affected in a manner that facilitates production of said polypeptide comprising;
 - i growing said strain under conditions conducive to the production and secretion of said polypeptide(s); and
 - 10 ii. recovering said polypeptide(s) from growth media and/or said bacterial strain.
2. A method according to Claim 1 wherein said microbial strain is a Gram positive strain.
3. A method according to Claims 1 or 2 wherein said microbial strain is 15 of the genus *Bacillus*.
4. A method according to Claim 1-3 wherein said bacterial strain is selected from *B.subtilis*, *B.amyloliquifaciens*, *B.licheniformis*, *B.stearothermophilus*.
5. A method according to Claim 1-4 wherein said polypeptide is selected 20 from carbonyl hydrolase, carbohydrase, protease, lipase, amylase, cellulase, oxidoreductase, glucoamylase or esterase.

6. A bacterial strain wherein a *wprA* gene has been altered by deletion and/or insertion and/or mutation and/or substitution so that the production and/or function of the gene product encoded by said *wprA* gene is prevented to the extent that the production of native, heterologous or recombinant 5 protein is facilitated.

7. A bacterial strain according to Claim 6 wherein said bacterial strain, before said alteration, is wild type for said *wprA* gene.

8. A bacterial strain according to Claims 6 or 7 wherein said bacterial strain is a Gram positive strain.

10 9 A bacterial strain according to Claims 6-8 wherein said bacterial strain is of the genus *Bacillus*.

10 A bacterial strain according to any proceeding Claim wherein said alteration is an insertion of genetic material into said *wprA* gene to prevent expression of said gene or to prevent synthesis of at least part of the 15 functional protein product.

11. A bacterial strain according to Claims 6-9 wherein said *wprA* gene is mutated by at least one selected point mutation to prevent expression of either the *wprA* gene or the WprA protein.

12 A bacterial strain according to Claims 6-9 wherein said alteration is to 20 an expression control sequence.

13. A bacterial strain according to Claim 12 wherein said expression control sequence is a promoter.

14. A bacterial strain according to Claim 13 wherein said alteration results in the provision of an inducible promoter to selectively control expression of the *wprA* gene.
15. A bacterial strain according to Claims 6-9, wherein said bacterial strain is *B.subtilis*, containing a deletion of at least part of the sequence represented in Figure 1 from nucleic acid base +154 to +247.
16. A bacterial strain according to Claim 15 wherein said *Bacillus* strain is deleted for part of the *wprA* gene encoding the signal sequence of the WprA precursor protein.
17. A bacterial strain according to Claims 6-9, wherein said bacterial strain is *B.subtilis*, containing a deletion of at least part of the sequence represented in Figure 1 from nucleic acid base +154 to +1392.
18. A bacterial strain according to Claims 6-9 or 17 wherein said deletion comprises at least part of the sequence represented in Figure 1 from nucleic acid base +247 to +1392.
19. A bacterial strain according to Claims 18 wherein the *wprA* gene is deleted for at least part of the *wprA* gene encoding CWPB23.
20. A bacterial strain according to Claims 6-9 wherein said bacterial strain is *B.subtilis*, containing a deletion of at least part of the sequence represented in Figure 1 from nucleic acid base +1392 to +2835.
21. A bacterial strain according to Claim 20 wherein the *wprA* gene is deleted for at least part of the *wprA* gene encoding CWPB52.

22. A bacterial strain according to Claims 20 or 21 wherein the *wprA* gene is deleted for at least part of the *wprA* gene encoding a serine protease.

23. A bacterial strain according to Claims 6-9 wherein said bacterial strain is *B.subtilis*, containing a deletion of at least part of the sequence 5 represented in Figure 1 from nucleic acid base +247 to + 2835.

24. A bacterial strain according to Claim 23 wherein said bacterial strain is deleted for part of the *wprA* gene encoding either or both the propeptide or protease.

Figure 1

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AAC	CCG	GCT	CTT	TGA	TAG	AGC	TGG	TTT	TTA	TAT	TTA	TCC	CCT	CAT	ATT	CCA	AAT	CAT	60		
TTA	AAA	TAA	CCT	TAA	AAT	TCC	CTG	TAA	GCG	GTA	TCT	CGT	CCT	ATG	AAA	TTA	TGA	TAC	120		
CAA	GGA	GAT	TCA	TCA	TTA	TTT	TGC	AGG	AGG	GAT	AAC	ATG	AAA	CGC	AGA	AAA	TTC	AGC	180		
M	K	R	R	K	F	S	S	S	V												
GTG	GCG	GCA	GTG	CTT	ATT	TTT	GCA	CTG	ATT	TTC	AGC	CTT	TTT	TCT	CCG	GGA	ACC	AAA	GCT	240	
V	A	A	V	L	I	F	A	L	I	F	S	L	F	S	P	G	T	K	A		
GCA	GGG	GCC	GGC	GCG	ATC	GAT	CAG	GCG	GCT	CTG	GAA	AAC	GGC	AAA	GAG	CAG	ACA	GGC	300		
A	A	A	G	A	I	D	Q	A	A	A	L	E	N	G	K	E	Q	T	G		
GCC	ATG	AAG	GAG	CCG	GAA	CAG	GTG	AAA	TGG	TAC	AAA	GTG	ACC	CCG	GGA	GCA	ACG	GAT	360		
A	M	K	E	P	E	Q	V	K	W	Y	K	V	T	P	G	A	T	D	I		
CAG	AAA	AAC	TCA	CAT	ATG	GCA	CTG	ACC	GTA	AAG	AGT	GAT	TCA	GTA	CTG	AAT	GTA	TCT	GTA	420	
Q	K	N	S	H	M	A	L	T	V	K	S	D	S	V	L	N	V	S	V		
TAT	CCA	AGT	AAG	GAA	AAA	GCG	CTT	AAA	GAT	GAG	ACG	TTT	GAG	ATG	TAC	CGT	TCT	TTC	ACA	480	
Y	P	S	K	K	E	K	A	L	K	D	E	T	F	E	M	Y	R	S	F	T	
GCG	GAG	GAT	GGA	AAA	AGC	GAA	GTC	ATT	TTT	CCA	TAC	GCG	TGG	AGC	GGC	CCT	TAC	TAT	GTA	540	
A	E	D	G	K	S	E	V	I	F	P	Y	A	W	S	G	P	Y	Y	V		
AAA	GTT	GAA	TAC	CTC	GGA	GAA	GAA	CCA	GAG	GAC	GGC	GGA	ACG	GCA	GAA	GCA	GCT	GCA	600		
K	V	E	Y	L	G	E	E	P	E	D	G	G	T	A	E	A	A	A			
GAA	GCC	AAG	TAT	ACG	ATT	GGG	TAT	AAA	GGC	ACC	AAA	CAG	CCG	TCA	GAT	TTA	GAA	GAG	660		
E	A	K	Y	T	I	G	Y	K	G	T	K	K	Q	P	S	D	L	E	E		
GAA	GAA	GCT	TGT	CCG	GTT	GAA	ATG	AGT	GTC	GAT	CAG	AG	AAA	TCA	GGA	AAA	GGC	ATC	CTG	720	
E	E	A	C	P	V	E	M	S	V	D	Q	K	K	S	G	K	G	I	L		
GAT	AAG	TTG	AGA	TCG	ATT	CGT	GAT	GAG	CAG	CTG	AGC	CAA	ACA	GCA	GAA	GGC	AAA	GAA	CTG	780	
D	K	L	R	S	I	R	D	E	Q	L	S	Q	T	A	E	G	K	E	L		
ACA	AGC	CTT	TAT	TAC	AAA	GCA	GCA	CCG	TTT	ATC	GTT	GCA	AAG	CTC	GCA	CTC	AAT	AAA	ACA	840	
T	S	L	Y	Y	K	A	A	P	F	I	V	A	K	L	A	L	N	K	T		
GCA	AGA	AAT	GAA	ATC	TAT	CAG	GAT	CTT	GTG	ACA	TTA	AAG	CCG	TTA	TTT	GAC	GAT	GTG	TCA	900	
A	R	N	E	I	Y	Q	D	L	V	T	L	K	P	L	F	D	D	V	S		
GAA	AAC	GGA	GCA	TCA	TCA	TCT	TCT	TAT	AAG	GTC	ACT	GAA	AAG	GAT	CAA	AAA	GCG	ATC	AAC	960	

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Figure 1-2 continued

E	N	G	A	S	S	S	Y	K	V	T	E	K	D	Q	K	A	I	N	R
CTA	TAT	GAT	AAA	GCT	TTA	CAA	TCA	GTC	CCG	TCA	TTC	CTT	AAA	GAG	GAG	ATA	AAG	AAA	CAA
L	Y	D	K	A	L	Q	S	V	P	S	F	L	K	E	E	I	K	K	Q
GGC	GAC	CGA	CTA	AAT	ATG	AAG	CAG	CTG	CAA	GGC	AAA	ACA	GCA	GGA	GCC	ATT	TTA	ACA	GAA
A	D	R	L	N	M	K	Q	L	Q	G	K	T	A	G	A	I	L	T	E
AAC	AAT	ATT	GCA	GCA	AAA	AGT	GAA	GTT	CAG	ACA	ACA	AAG	GTT	ATT	TTC	AAG	GTC	AAG	GAC
N	N	I	A	A	K	S	E	V	Q	T	T	K	V	I	F	K	V	K	D
AAT	AAA	AGC	CTC	TCA	TCC	GTA	CAT	AAT	GAA	ATG	AAG	GGC	TTT	TCT	GCA	AGC	GCG	CAA	TCG
N	K	S	L	S	V	H	N	E	M	K	G	F	S	A	S	A	Q	S	
AAA	AAA	GAC	ATA	TCT	AAT	GTG	AAA	AAG	GCA	AAG	AAA	CTG	TTT	GAC	AAT	CTG	TAT	TCA	TTT
K	K	D	I	S	N	V	K	K	A	K	K	L	F	D	N	L	Y	S	F
GAA	CTT	CCG	AAA	GAC	GAG	AAA	CAG	AAC	GGC	GCA	TAT	ACG	GCA	AGC	GCC	AAA	AGG	GTC	AAA
E	L	P	K	D	E	K	Q	N	G	A	Y	T	A	S	A	K	R	V	K
AGC	GCT	GCT	GGG	ACA	CTA	TCC	AAG	ATG	TCC	AAAT	GTA	GAG	TTT	GCG	GAA	CCC	GTA	CAG	GAA
S	A	A	A	T	L	S	K	M	S	N	V	E	F	A	E	P	V	Q	E
TAC	AAA	AGC	TTG	GCA	AAC	GAT	ATT	CAG	TAC	CCT	TAT	CAA	TGG	CCG	CTT	AAA	AAC	AAC	GGT
Y	K	S	L	A	N	D	I	Q	Y	P	Y	Q	W	P	L	K	N	N	G
GAA	AAC	GGC	GGT	GTC	AAA	AAT	GCG	GAT	GTG	AAA	TAT	GAG	CCT	GCC	AAC	ACA	TTG	CTG	TCC
E	N	G	G	V	K	N	A	D	V	K	Y	E	P	A	N	T	L	L	S
AAA	CGC	AAG	CTT	AAC	GAT	ACA	CTC	ATT	GCA	GTA	GTA	GAC	ACA	GGC	GTA	GAC	AGC	ACG	CTT
K	R	K	L	N	D	T	L	I	A	V	V	D	T	G	V	D	S	T	L
GCC	GAT	TTA	AAA	GGA	AAA	GTA	AGA	ACA	GAT	CTC	GGG	CAC	AAT	TTT	GTC	GGG	CGA	AAT	AAC
A	D	L	K	G	K	V	R	T	D	L	G	H	N	F	V	G	R	N	N
AAT	GCA	ATG	GAT	GAT	CAG	GGG	CAT	GGG	ACG	CAT	GTC	GCA	GGC	ATT	ATT	GCA	GCC	CAA	AGC
N	A	M	D	D	Q	G	H	G	T	H	V	A	G	I	I	A	A	Q	S
GAT	AAC	GGC	TAT	TCA	ATG	ACT	GGG	TTG	AAT	GCC	AAA	GCA	AAA	ATC	ATC	CCT	GTA	AAA	GTG
D	N	G	Y	S	M	T	G	L	N	A	K	A	K	I	I	P	V	K	V
CTT	GAT	TCC	GCA	GGT	TCC	GGA	GAT	ACT	GAA	CAA	ATT	GCT	CTC	GGC	ATC	AAA	TAT	GCT	GCT
L	D	S	A	G	S	G	D	T	E	Q	I	A	L	G	I	K	Y	A	A
GAC	AAA	GGA	GCA	AAG	GTG	ATT	AAT	TTA	AGT	TTA	GGC	GGG	TAC	AGC	CGC	GTG	CTT	GAA	1860

Figure 1-3 continued

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D	K	G	A	K	V	I	N	L	S	L	G	G	Y	S	R	V	L	E
TTT	GCT	TTC	AAG	TAC	GCA	GCT	GAC	AAA	AAT	GTC	TTG	ATT	GCC	GCC	AGC	GGG	AAT	GAT
F	A	L	K	Y	A	A	D	K	N	V	L	I	A	A	S	G	N	D
GGA	GAA	AAT	GCC	TTA	TCT	TAT	CCT	GCA	TCT	TCT	AAA	TAT	GTG	ATG	TCA	GTC	GGC	GCA
G	E	N	A	L	S	Y	P	A	S	S	K	Y	V	M	S	V	G	A
AAT	CGC	ATG	GAT	ATG	ACC	GCT	GAT	TTC	TCT	AAC	TAT	GGA	AAA	GGT	CTG	GAC	ATC	TCT
N	R	M	D	M	T	A	D	F	S	N	Y	G	K	G	L	D	I	S
CCA	GGG	TCT	GAT	ATC	CCG	AGC	TTA	GTG	CCG	AAC	GGG	AAT	GTC	ACG	TAC	ATG	AGC	ACG
P	G	S	D	I	P	S	L	V	P	N	G	N	V	T	Y	M	S	G
TCT	ATG	GGC	ACG	CCA	TAT	GCT	GCC	GCT	GGG	GGG	CTG	CTG	TTT	GCT	CAA	AAT	CCT	AAG
S	M	A	T	P	Y	A	A	A	A	A	G	L	F	A	Q	N	P	K
CTA	AAA	AGA	ACA	GAA	GTT	GAG	GAT	ATG	TTG	AAA	AAG	ACG	GCA	GAT	GAT	ATT	TCC	TTT
L	K	R	T	E	V	E	D	M	L	K	T	A	D	D	I	S	F	E
AGT	GTC	GAT	GGC	GGG	GAA	GAA	GAG	GAG	TTC	TAT	GAC	GAT	TAT	GGC	GAT	CCG	ATT	GAA
S	V	D	G	G	E	E	L	Y	D	D	Y	G	D	P	I	E	I	P
AAG	ACA	CCT	GGT	GTA	GAC	TGG	CAT	TCA	GGC	TAC	GGG	CGG	CTG	AAT	GTC	ATG	AAG	GCT
K	T	P	G	V	D	W	H	S	G	Y	G	R	L	N	V	M	K	A
AGC	GCA	GCT	GAT	TTA	CAG	CTT	AAG	GTC	AAC	AAG	CTG	GAA	AGC	ACT	CAA	ACA	GCT	AGA
S	A	A	D	L	Q	L	K	V	N	K	L	E	S	T	Q	T	A	V
GGA	AGC	GCG	AAG	GAA	GGC	ACC	CTT	ATC	GAG	GTG	ATG	AAC	GGC	AAA	AAG	AAA	CTC	GGC
G	S	A	K	E	G	T	L	I	E	V	M	N	G	K	K	L	G	S
GCC	AAA	GCC	GGG	AAA	GAC	AAT	GCG	TTC	AAG	GTG	AAT	ATC	GGG	ACT	CAA	AAA	CAG	GAT
A	K	A	G	K	D	N	A	F	K	V	N	I	A	T	Q	K	Q	D
GTA	CTG	TAT	CTG	AAA	GCA	ACA	AAA	GGC	GAT	GGC	AAA	ACA	TCG	TAT	AAA	GTT	GTC	GTC
V	L	Y	L	K	A	T	K	G	D	A	K	T	S	Y	K	V	V	V
AAA	GGA	AAA	CCT	TCC	GGC	ACA	CCG	AAA	GTA	ARC	GGG	GTG	AAA	ACG	AAG	GAT	ACG	GCA
K	G	G	K	P	S	G	T	P	K	V	N	A	V	K	T	K	D	T
AAA	GGG	AAG	GCA	AAC	AGC	AAA	GCG	ATG	ATC	AGA	GTG	AAA	AAC	AAA	TCA	AAG	AAA	GTC
K	G	K	A	N	S	K	A	M	I	R	V	K	N	K	S	K	K	V
GCT	TCT	GCC	AAA	GCT	GAC	GCA	AAA	GGA	ACG	TTG	GTG	AAA	ATC	AAA	CAA	AAA	GCC	2760

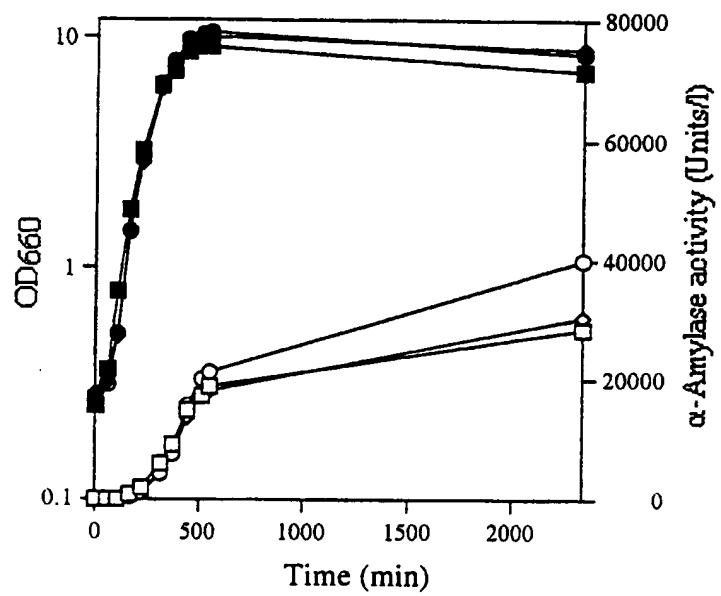
Figure 1-4 continued

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A	S	A	K	A	D	A	K	G	T	F	S	V	K	I	K	K	Q	K	A		
GGA	ACG	GTG	CTG	TAC	GTC	ACG	GCT	GTG	GAT	ACA	GAT	AAA	AAA	GAA	AGC	AGC	AAG	GAA	GCA	AAA	2820
G	T	V	L	Y	V	T	A	V	D	T	D	K	K	E	S	K	E	A	K		
GTT	GTT	GTT	GAA	AAG	TAA	CCA	AAA	AGC	GGT	GCT	CGA	TGC	ACC	GCT	TTT	TTA	TTT	GCG	CCC	2880	
V	V	V	E	K																	
CCG	TTC	GAC	TGC	TGT	GTA	CAT	AAT	GGA	TCG	CTT	TCC	GTT	TGA	AGC	CGT	CCA	TTC	AAA	ACG	2940	
GTC	TCC	TGT	CCC	TTC	GCT	GCT	GTA	GTT	GTA	GTG	ATG	CTG	CAT	CGC	GCA	TTG	TGC	GGT	GTC	3000	
ATT	TGA	AAA	GTG	ATA	AAA	GGA	CAC	ACC	CTG	CCC	CGC	CGA	AGT	ATT	CGA	AAA	GAC	TCC	TTT	3060	
ATT	ATA	AGA	AAA	GAC	GCA	ATC	GAA	TAC	CGA	TAA	GCC	GAA	GCT	GTG	CAA	GTG	CTG	CAG	3117		

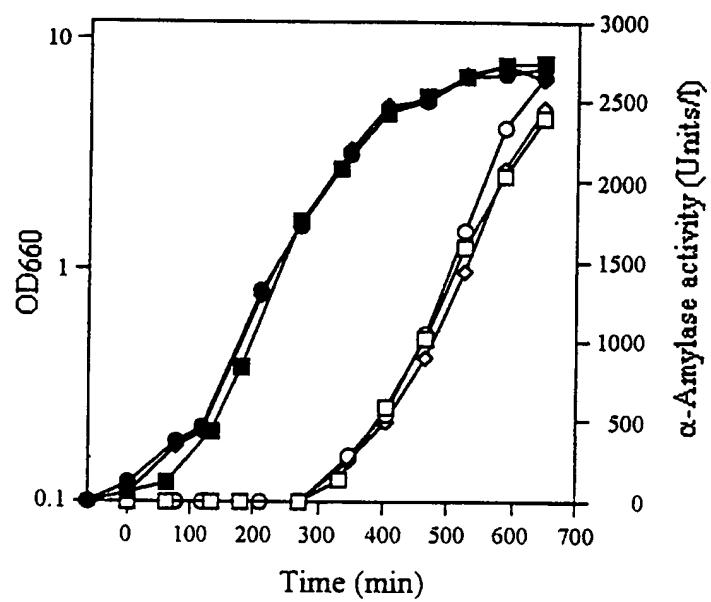
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Figure 2A



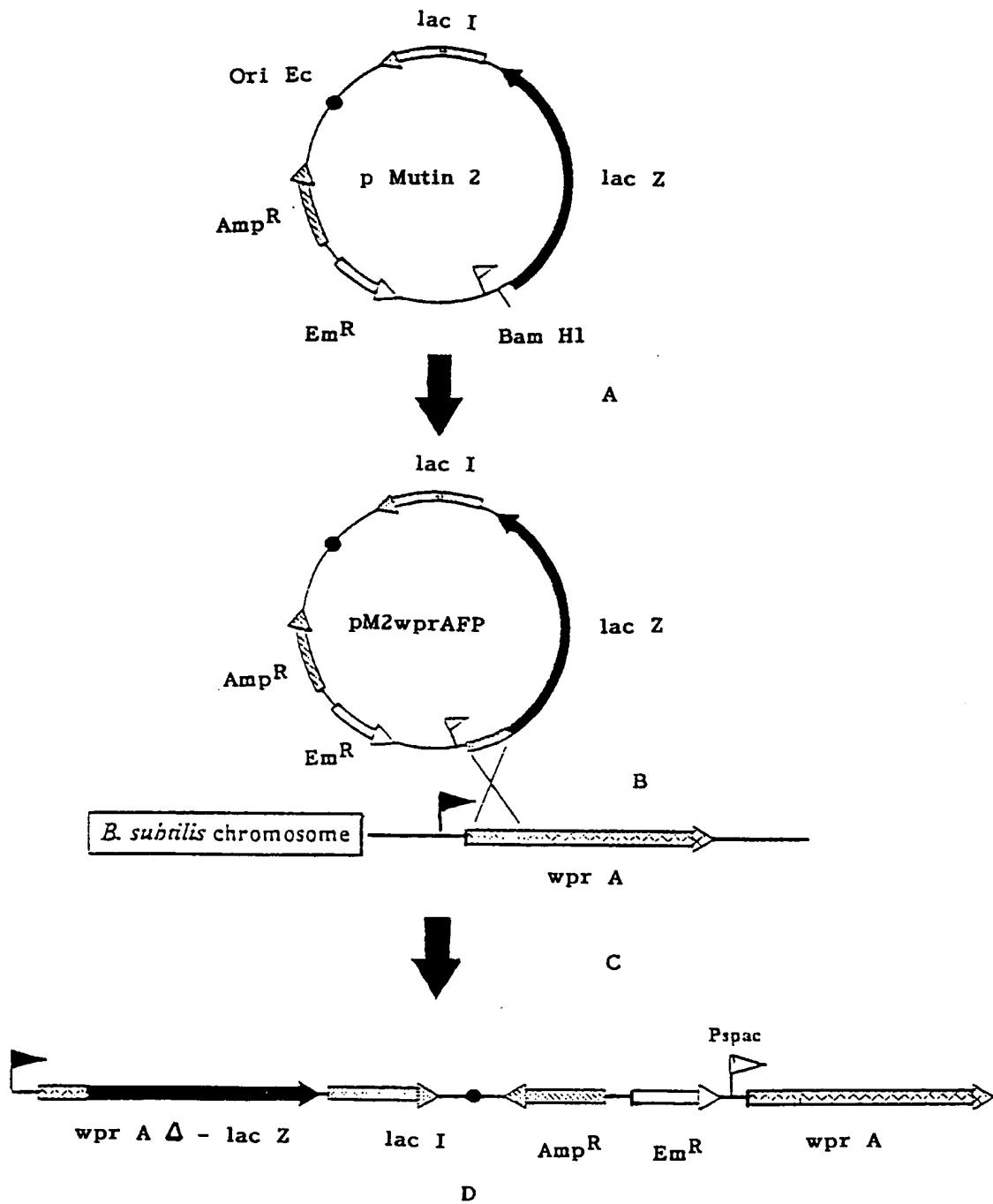
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Figure 2B



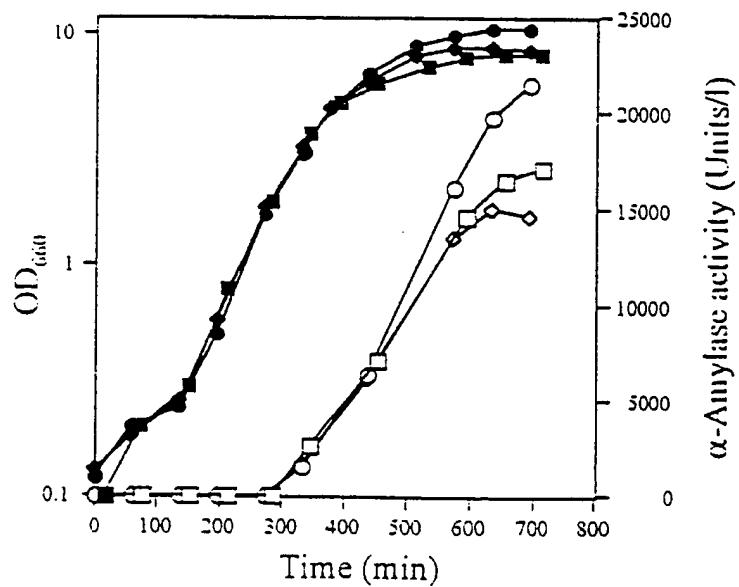
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Figure 2C

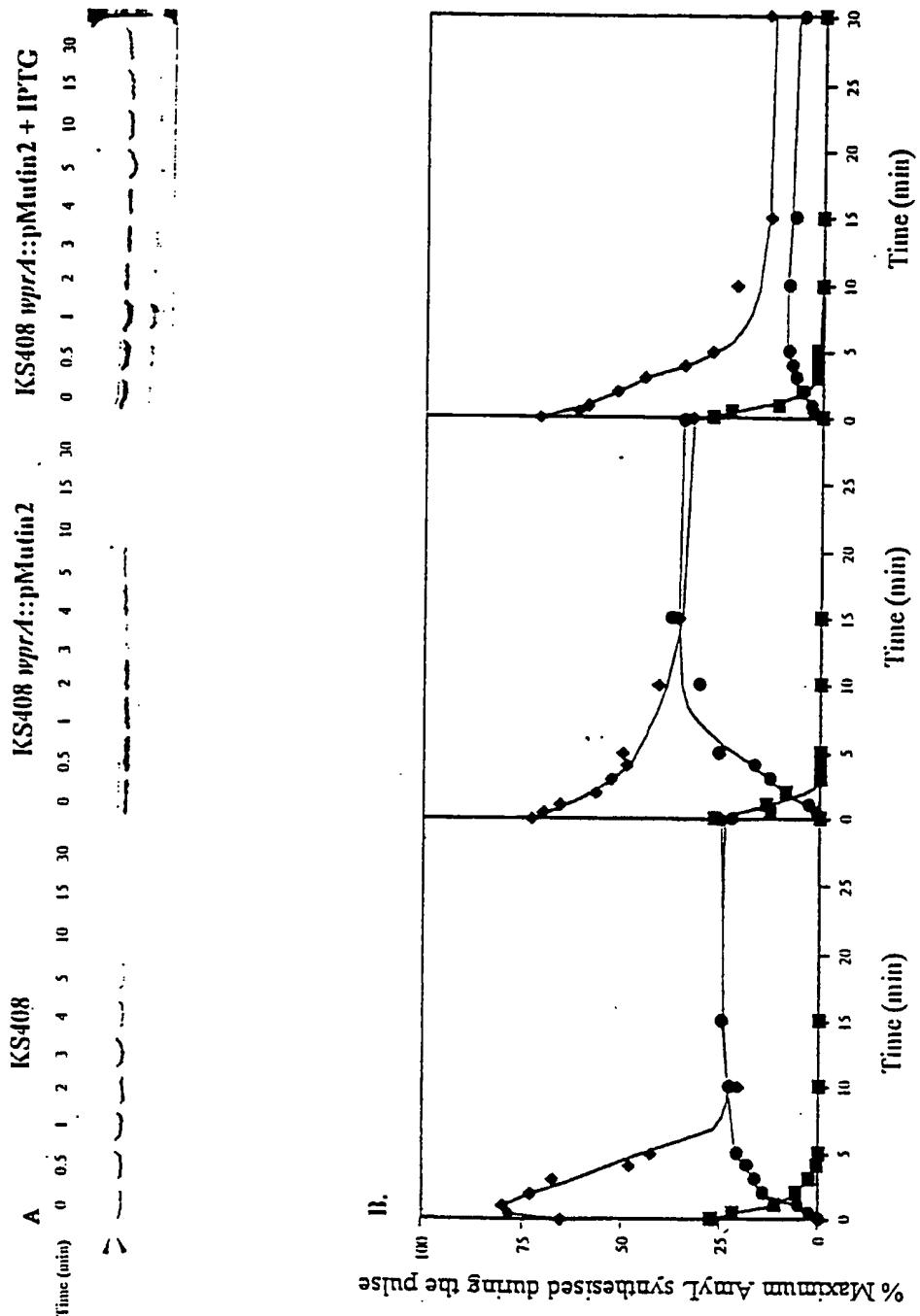


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Figure 3

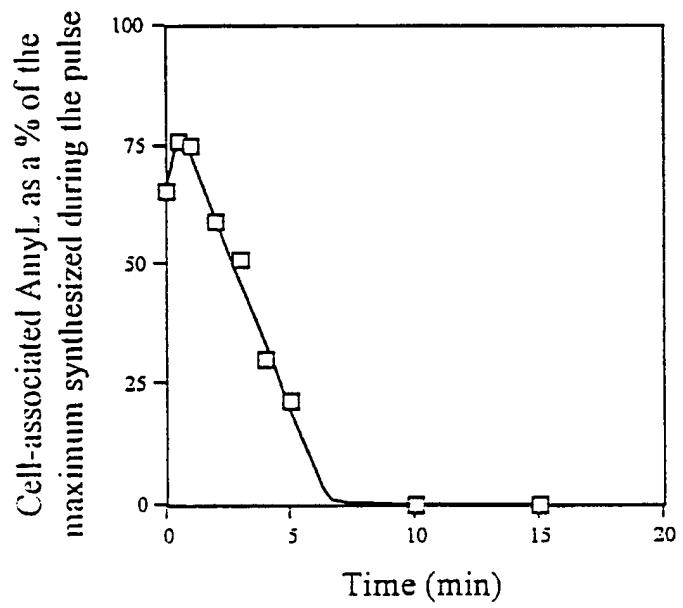


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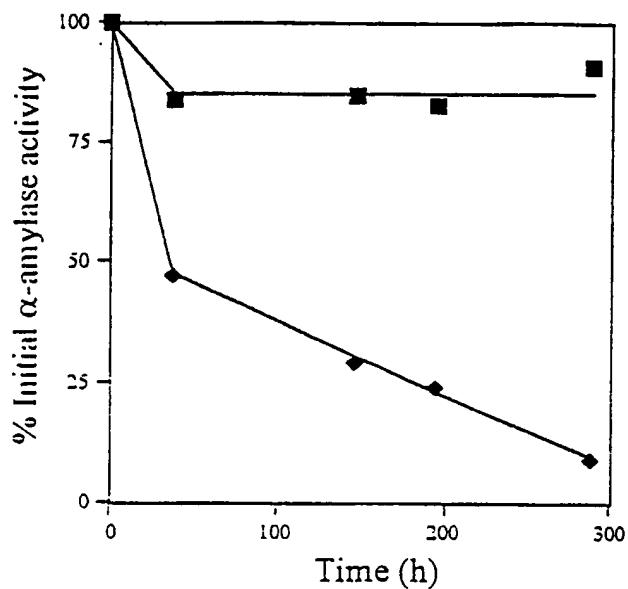
Figure 5



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Figure 6

A.



B.

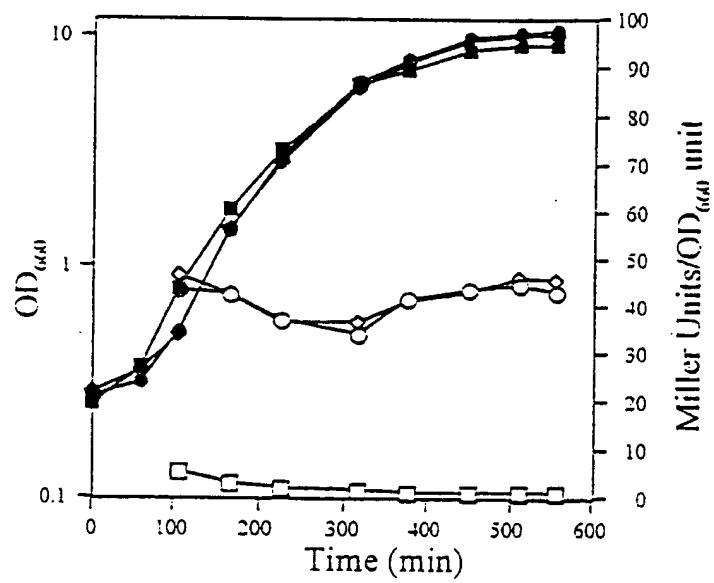
Time (h) 0 38 146 194 288

- EDTA

+ EDTA

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Figure 7



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/01051

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/75 C12N9/28 C12N1/21 C12R1/125 //C12N9/56

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ^a	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MARGOT P ET AL: "The wprA gene of <i>Bacillus subtilis</i> 168, expressed during exponential growth, encodes a cell-wall-associated protease." <i>MICROBIOLOGY (READING)</i> 142 (12). 1996. 3437-3444. ISSN: 1350-0872, XP002075685 see the whole document ---	1-24
Y	SIMONEN M ET AL: "PROTEIN SECRETION IN <i>BACILLUS</i> SPECIES" <i>MICROBIOLOGICAL REVIEWS</i> , vol. 57, no. 1, 1 March 1993, pages 109-137, XP000562630 see page 121, right-hand column - page 122, left-hand column ---	1-24 -/-

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Date of the actual completion of the international search	Date of mailing of the international search report
27 August 1998	09/09/1998
Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 369 817 A (BIOTEKNIKA INTERNATIONAL) 23 May 1990 see the whole document ----	1-24
Y	WO 92 16642 A (OMNIGENE INC) 1 October 1992 see the whole document ----	1-24

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 98/01051

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		CA 2003078	A	18-05-1990
		DE 68926333	D	30-05-1996
		DE 68926333	T	28-11-1996
		DK 577989	A	19-05-1990
		JP 3067582	A	22-03-1991
		US 5589383	A	31-12-1996
		US 5620880	A	15-04-1997
WO 9216642	A 01-10-1992	US 5294542	A	15-03-1994
		EP 0576606	A	05-01-1994

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